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New and Notable

A Molecular Voltmeter Based on Fluorescence Dynamics

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INTRODUCTION

In this issue, Hou et al. (1) introduce an elegant method for measuring the absolute voltage across a cell membrane with an accuracy of 10 mV. Their study presents an incisive approach to voltage imaging in which the voltage value is encoded in the temporal dynamics of fluorescence rather than in the raw fluorescence intensity. Together with an engineered microbial rhodopsin with properties amenable to the approach, the method provides a genetically encoded optical read-out independent of the reporter's expression level and insensitive to variations in common experimental parameters. Their work presents a conceptually new avenue toward developing probes for membrane voltage useful for myriad systems from bacteria to mammalian tissue.

THE CHALLENGE

Membrane electrical potentials are ubiquitous and every protein, lipid, and small molecule embedded in a membrane is influenced by the associated electric field. Nevertheless, our understanding of the biophysics of membrane potential is heavily biased toward a few systems that are easiest to measure, such as fast action potentials in excitable cells and chemiosmotic coupling in prokaryotes and organelles derived from them. There will undoubtedly be surprising new roles of membrane voltage in the vast array of relatively unexplored fungal, plant, and animal cells, and in processes in which membrane potential is implicated, such as embryonic development, wound healing, and metabolic regulation in prokaryotic and eukaryotic cells. Accurate voltage measurements will be essential for such studies.

Voltage-sensitive genetically encoded fluorescent proteins have been used to monitor changes in membrane electrical potential in cells for over a decade, and in the past few years probe sensitivity and response times have reached spectacular levels enabling single action potentials in individual neurons to be directly visualized (see references 11–16 in Hou et al.). However, these intensity-based optical reporters, valuable for recording rapid voltage changes, are not reliable for measurement of the absolute membrane voltage value. Uncertainties in the concentration of properly folded reporter protein and extent of trafficking to the plasma membrane, photobleaching, and background autofluorescence, all work against the accuracy and reproducibility needed to measure absolute resting voltage and its slow and more subtle alterations during physiological transformations. Varying efficiencies of probe light delivery and emitted light collection also frustrate attempts to use fluorescence intensity measurements for quantification of membrane voltage, especially when physiological changes are accompanied by changes in morphology or sample movement. For example, spatial differences in membrane potential are critical factors in embryogenesis and complex tissue regeneration (2), but to map these differences throughout development means the investigator is confronted with a continuously changing sample. Genetically encoded fluorescent reporters of calcium have recently been shown to be an effective means to monitor neural activity in vivo and in vitro (3), but calcium transients are much slower than neuronal action potentials and do not provide direct voltage information.

TIME-DOMAIN VERSUS INTENSITY-DOMAIN CODING

What is needed is an optical voltage reporter insensitive to the experimental parameters noted above and also insensitive to the precise intensity of illumination and the efficiency of optical collection, which is extremely difficult to maintain constant in morphologically complex tissue. Hou et al. (1) reasoned that they could design such a reporter by encoding the membrane potential values in a time-domain function, i.e., a kinetic measure reflecting the rate of a membrane voltage-dependent transition, rather than an intensity-based parameter.

The logic favoring time-domain fluorescence encoding over intensity encoding is similar to the reason FM radio is less subject to noise than AM radio (Fig. 1). In an amplitude-modulated (AM) transmission, the amplitude of the radio waves reaching a receiver can be distorted by intervening clouds, trees, and birds. The receiver cannot distinguish this amplitude noise from real signal, so sound fidelity is low. In a frequency-modulated (FM) transmission, the audio information is encoded in the time course of the radio signal, not its amplitude, so the receiver can readily distinguish the real signal from amplitude noise. In general, measurements in the time domain are more precise and accurate than measurements of amplitude.

Following this logic, Hou et al. (1) based their optical voltmeter not on fluorescence intensity but on fluorescence dynamics deriving from the voltage-dependent kinetics of spectral transitions in the photochemical reaction cycle of a microbial rhodopsin.

MICROBIAL RHODOPSINS TO THE RESCUE

Microbial rhodopsins comprise a large family of photoactive membrane proteins (as of this writing, ~7000 genes

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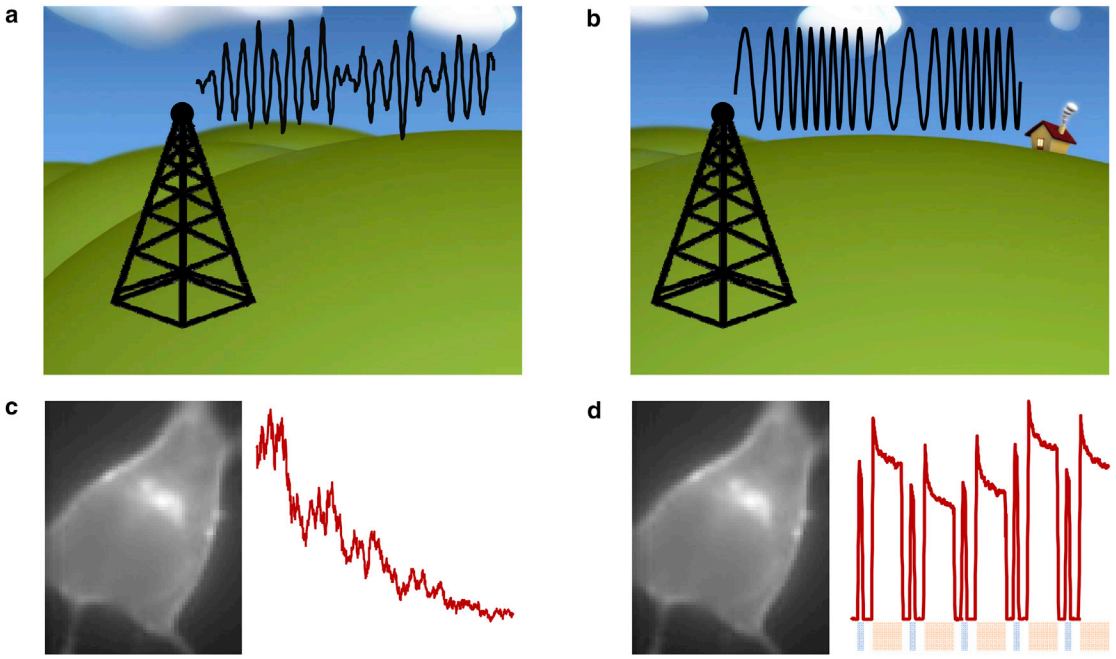

FIGURE 1 Time-domain encoding increases measurement accuracy. The new voltage indicator reported by Hou et al. (1) encodes voltage in the kinetics of a fluorescence transient, thereby achieving greater absolute accuracy than is possible with reporters that encode information in fluorescence intensity alone. (*a*) In an AM radio broadcast, spurious fluctuations in the amplitude of the signal contribute to noise at the receiver. (*b*) In an FM radio broadcast, the information is encoded in the time domain, so amplitude noise is easily removed by the receiver. (*c*) In a conventional fluorescent reporter, spurious contributions to the fluorescence intensity arise from photobleaching, movement of the sample, background autofluorescence, and variations in illumination intensity and detector efficiency. (*d*) In the absolute voltage indicator of Hou et al. (1), pulses of blue light drive a microbial rhodopsin into a nonequilibrium state and the kinetics of relaxation under orange light reports the absolute membrane voltage. (Figure courtesy of Adam E. Cohen.) To see this figure in color, go online.

are known) with a variety of functions throughout the microbial world (4). Their seven transmembrane helices form a pocket for the chromophore retinal attached in a protonated Schiff base linkage in the protein's core. Photoisomerization of retinal causes structural changes in the protein that result in ion transport or sensory signaling by a variety of mechanisms. Their simplicity as single polypeptides, ability to fold properly in membranes of very different lipid composition, their diversity in function, and wide range of absorption maxima make them attractive as genetically encoded optical tools. Importantly, retinal spontaneously binds and correctly attaches to the apoprotein when added exogenously or when present in the target cell as a natural metabolite (as in neurons). In addition, microbial rhodopsins, unlike higher animal visual pigments, exhibit a cyclic series of photochemical reactions, i.e., a photocycle, producing spectrally distinct intermediates that return spontaneously to the dark state enabling repetitive activation without loss of activity.

After decades of research on their diversity, structure, photochemistry, and function, selected microbial rhodopsins are beginning to be used as light-triggered tools to gain optical control over functions in cells that do not normally express them, a technology called optogenetics (5). Most notably, channelrhodopsin-2, an algal phototaxis receptor that uses light to depolarize the plasma membrane (6) and acts as a light-gated cation channel when expressed in animal cells (7), is widely used for the light-triggering of action potentials in neurons. Archaerhodopsin-3 (Arch), a light-driven proton pump used by haloarchaea to capture light energy (8), hyperpolarizes neuron membranes when illuminated, thereby suppressing neuronal firing (9). The use of these and other microbial rhodopsins as optogenetic tools has led to a revolution in study of the brains of model organisms, including worms, flies, zebrafish, and mice (10).

In 2011 and 2012, a pair of articles from Kralj et al. (11,12) showed that microbial rhodopsin proton pumps can be used as fluorescent indicators of membrane potential as well as for optogenetic photocontrol. The team demonstrated this effect first with proteorhodopsin in bacteria (11), and then with Arch in mammalian neurons (12). Rhodopsin-based voltage indicators are exquisitely sensitive to membrane potential and fast, but also very dim. A subsequent mechanistic study revealed that the voltage sensitivity did not arise in the dark state of the proteins. Rather, the bright light used for imaging established a photo-steady-state distribution of photocycle intermediates. Membrane voltage shifted this nonequilibrium distribution

between a fluorescent state and a manifold of nonfluorescent states (13). This finding led Hou et al. (1) to introduce their conceptually new approach to absolute voltage measurements, which is based on the complex photocycle kinetics of Arch. They hypothesized that in the kinetics of relaxation from one nonequilibrium steady state to another, the time course of the fluorescence might encode absolute membrane voltage. Such a measurement would be insensitive to the sources of noise that plague intensity-based measurements. They generated mutants of Arch, expressed them in HEK293 cells, used a patch pipette to set the membrane voltage, and applied illumination pulses of varying wavelength, intensity, and timing, while monitoring the fluorescence for voltage-dependent transients. Through this systematic search, they identified a mutant, Arch(D95H), and an illumination protocol that together encoded absolute voltage in fluorescence transients lasting tens of milliseconds.

The trick developed by Hou et al. comes at a cost: by using the time domain to encode voltage information, the technique sacrifices the temporal resolution required to monitor rapidly varying potentials. In the scheme of Hou et al. (1) each voltage measurement involves an illumination pulse sequence that lasts nearly 2 s, and to achieve an accuracy of 10 mV, they average over 6–12 such measurements. A challenge for future sensors of this type will be to improve the brightness and measurement speed to achieve higher measurement bandwidths at greater accuracy. Such improvement may well come from extending the approach used with Arch to other microbial rhodopsins, which offer countless variations in their photocycle transitions and kinetics.

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